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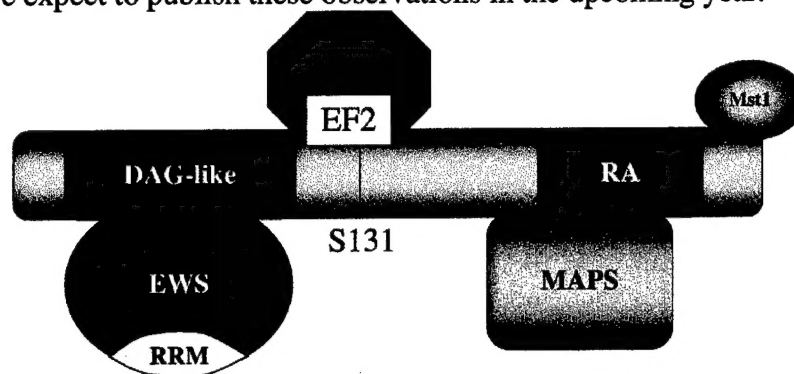
Introduction:

The subject of this research is the RASSF1 locus located at 3p21. Our group and others have found that this locus expresses two related gene products, RASSF1A and RASSF1C. RASSF1A expression is lost at high frequency in a wide range of tumor types, primarily through hyper methylation of the RASSF1A-specific promoter. The purpose of this work was to explore the possibility that RASSF1A protein has important tumor suppressor activity. We employed gain-of-function and loss-of-function analysis to examine the contribution of RASSF1A to tumor cell growth and survival.

Body:

Task 1: Analysis of functional consequences of RASSF1A and RASSF1C expression in breast cancer derived cell lines. As described in the previous report, this task has been completed (Burbee et al., 2001). We found that RASSF1A but not RASSF1C can revert tumorigenic phenotypes.

Task 2: To characterize the molecular physiology of RASSF1A. As described in the previous report, we have found that RASSF1A is a physiologically relevant negative regulator of cell cycle progression and this likely accounts for its tumor-suppressive activity (Shivakumar et al., 2002). We have demonstrated that RASSF1A inhibits cell cycle progression through inhibition of Cyclin D1 translation, and we have moved on to characterize the molecular connection between RASSF1A and this activity. During the past year we have identified and validated several RASSF1A-interacting proteins that establish links between RASSF1A and cyclin D1 translational machinery. These physical interactions suggest that RASSF1A can establish translational dormancy on cyclin D1 mRNA by assembling an inhibitory complex consisting of the RNA-binding protein EWS, and a novel EFG homolog that lacks a GTPase domain. We expect to publish these observations in the upcoming year.



Using a combination of yeast two-hybrid screens and validation by co-immunoprecipitation from HeLa cells and in vitro binding assays, we have found that RASSF1A interacts with EWS, MST1/2, and a novel EF2 homolog. We have begun a functional evaluation of these interactions. To date we find that inhibiting MST1/2 expression by RNAi will rescue RASSF1A-induced cell cycle arrest. In addition we have isolated several peptide aptamers that associate with RASSF1A in a manner that can inhibit MST1/2 interaction. We are currently testing the capacity of these aptamers to inhibit RASSF1A function in cells. Interestingly, it has recently been reported that RASSF1A expression is commonly inactivated in pediatric tumors, with the exception of Ewing's sarcoma. Given our observation of an EWS/RASSF1A complex, this presents an exciting genetic correlation that may indicate EWS may

function directly downstream of RASSF1A. We are evaluating this hypothesis by testing the consequences of EWS siRNA on RASSF1A-induced cell cycle arrest in H1299 cells. In addition, we have acquired several Ewing's sarcoma-derived cell lines. We will test the sensitivity of these lines to RASSF1A expression.

Key Training Accomplishments:

- Ms. Thomas has acquired technical expertise in tumorigenicity assays, protein-interaction assays, immunofluorescence analysis, and RNAi.
- Ms. Thomas has received training in hypothesis generation, hypothesis testing, and data acquisition and analysis.
- Ms. Thomas has learned the central importance of appropriate controls in the process of producing interpretable results.

Key Research accomplishments:

- Restoration of RASSF1A expression can revert tumorigenic phenotypes.
- RASSF1A negatively regulates Cyclin D1 translation.
- RASSF1A assembles a translational inhibitory complex on cyclin D1 mRNA.
- RASSF1A interacts with EWS.
- MST1/2 are required for the cytostatic activity of RASSF1A.

Reportable outcomes:

Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., *et al.* (2001). Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* 93, 691-699.

Shivakumar, L., Minna, J., Sakamaki, T., Pestell, R., and White, M. A. (2002). The RASSF1A Tumor Suppressor Blocks Cell Cycle Progression and Inhibits Cyclin D1 Accumulation. *Mol Cell Biol* 22, 4309-4318.

Shivakumar, L. (2002). RASF1A: A frequently targeted tumor suppressor that negatively regulates cell cycle progression. Ph.D. thesis. UT Southwestern Medical Center.

As a result of the training provided by this grant, Dr. Shivakumar is currently a Science Fellow at Aurigene Discovery Technologies.

Conclusions:

This work strongly suggests that RASSF1A is a tumor suppressor protein that is frequently lost in human cancer. Our observation of germline mutations that result in expression of defective RASSF1A proteins has the potential to aid future risk assessment. Continued work directed at defining the molecular nature of RASSF1A function will enhance our understanding of breast cancer development and progression, and may reveal novel entry points for therapeutic strategies.

References:

Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., *et al.* (2001). Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* 93, 691-699.

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